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The intracellular fate of an amphipathic pH-responsive polymer: key characteristics towards drug delivery

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Abstract

Biopolymers have become important drug delivery systems for therapeutic molecules by enhancing their accessibility and efficacy intracellularly. However, the transport of these drugs across the cell membrane and their release into the cytosol remain a challenge. The trafficking of poly (L-lysine iso-phthalamide) grafted with phenylalanine (PP-50) was investigated using an osteosarcoma cell line (SAOS-2). Colocalisation of this amphipathic biopolymer with endocytosis tracers, such as transferrin and lactosylceramide, suggested that PP-50 is partially internalised by both clathrin and caveolin-mediated endocytosis. Macropinocytosis was also investigated, but a smaller correlation was found between this mechanism and PP-50 transport. A significant decrease in polymer-mediated calcein uptake was found when cells were pre-incubated with endocytosis inhibitors, suggesting also the use of a combination of mechanisms for cell internalisation. In addition, PP-50 colocalisation with endosome and lysosome pathway markers showed that the polymer was able to escape the endolysosomal compartment before maturation. This is a critical characteristic of a biopolymer towards use as drug delivery systems and biomedical applications.

Key words

PP-50, amphipathic polymer, endocytosis mechanisms, caveolin, clathrin, macropinocytosis

1. Introduction

In the past years, biopolymers have become an attractive choice as drug delivery systems. Their role in this field has been long recognised, with controlled release of therapeutic drugs over long periods, easy conjugation for active targeting, cyclic dosage, and adjustable release of both hydrophilic and hydrophobic molecules [1,2].

Biopolymers can successfully transport a variety of molecules across mammalian cell membranes, such as cancer therapy candidates and proteins [3,4]. The transport of these polymers along different therapeutics to a particular site within cells has shown a drastic improvement in the accessibility and efficacy of drugs [5]. However, most of the transport of these carriers through the cell membrane ends trapped within the endolysosomal pathway, with both the carrier and delivery molecule unable to reach their target [6,7]. This issue has hindered the use of several biomolecules and has been recognised as a critical barrier to further advancement in the field [8,9].

Endocytosis is a complex internalisation process involved in the uptake of different molecules. Depending on the properties of the internalised molecules, this process can take place using different mechanisms, such as phagocytosis and pinocytosis. Phagocytosis is the cell engulfment of solid particles, a key mechanism of innate immunity, while pinocytosis is a receptor-mediated uptake of smaller particles present in all cell types [10,11]. Pinocytosis can be divided in three separate processes: macropinocytosis (MPC), clathrin-dependent (CDE), or clathrin-independent endocytosis (CIE). The caveolin-mediated pathway (CavME) is a subdivision of CIE.

For efficient intracellular delivery of therapeutics, delivery systems need to facilitate their release into the cytoplasm by the disruption of endosomal membranes under moderate acidic conditions (pH 5.0–6.8) [12,13]. One effective method to achieve this disruption involves the use of anionic amphipathic biodegradable pH-responsive polymers. These molecules undergo a conformational change from extended charged chains to aggregated hydrophobic structures as the environmental pH drops below their pKa, thus interacting with the hydrophobic interior of phospholipid bilayers. In low pH environments, the carboxylate groups become protonated and hydrophobic interactions within the polymer backbone generate a transition to a compacted lipid membrane disruptive state [14]. This transition has been manipulated by the grafting of L-phenylalanine on the pendant carboxylic acid, resulting in a maximal lytic activity at pH 6.5, a characteristic pH range of early endosomes [12].

PP-50, an amphipathic polymer, has shown recently to be able to transport hydrophilic molecules into cells and to be biocompatible [15,16]. Despite its successful use in cryopreservation, the trafficking of PP-50 through cell membranes has not been previously investigated in depth. In this study, the fate of PP-50 when entering the endocytic pathway was evaluated in osteosarcoma cells, a model for adherent cells, to provide key information regarding this amphipathic biopolymer towards use in cryopreservation and other biomedical applications.

2. Materials and methods

2.1. Materials

The SAOS-2 cells were obtained from the European Collection of Cell Cultures. Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were purchased from Invitrogen (UK). Dimethyl sulfoxide (Me₂SO) and sodium hydroxide (NaOH) were purchased from ThermoFisher (UK). Phosphate-Buffered Saline (PBS), trypsin–EDTA, Alexa Fluor 647 cadaverin, transferrin-AF488, lactosylceramide (lacCer)-BODIPY, fluorescein isothiocyanate–dextran, CellLight Early Endosomes-GFP, CellLight Late Endosomes-GFP, CellLight Lysosomes-GFP, and LysoTracker® Deep Red were purchased from Life Technologies™ (UK). The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was obtained from Promega (UK). Bafilomycin A1 (BafA1), methyl-beta-cyclodextrin (mβcd), nystatin, sucrose, rottlerin, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (sulfo-NHS), calcein, propidium iodide (PI) and Hoechst H33342 were obtained from Sigma-Aldrich (UK). Visking tubing was purchased from Medicell Membranes Ltd. (UK). All chemicals and biochemicals used were of analytical grade.

2.2. Polymer synthesis and characterisation

2.2.1. PP-50

The synthesis and characterisation of PP-50 were as previously described by Eccleston and Chen [17,18]. Briefly, PP-50 synthesis involved two sections: synthesis of the polymer backbone, and the amino acid grafting. The backbone consisting of poly (L-lysine *iso*-phthalamide) (PLP) was synthesised by a polycondensation reaction between L-lysine methyl ester dihydrochloride and *iso*-phthaloyl chloride to form poly (L-lysine methyl ester

iso-phthalamide). In order to remove the methyl group, the solution was dissolved in Me₂SO (0.5 M) and then mixed with 5 % (w/v) NaOH. The hydrolysed product precipitated and was collected by vacuum filtration and dissolved in deionised water. Dialysis purification and lyophilisation produced PP-50 in powder. The grafting of L-phenylalanine to the backbone was achieved by the use of standard DCC/DMAP mediated coupling techniques. PP-50 is used in cell culture as a solution at a concentration of 250 µg/ml.

2.2.2. PP-50/AF647

The conjugation of the dye Alexa Fluor 647 cadaverine (AF647) to PP-50 was achieved by standard EDC/sulfo-NHS amide reaction [19]. Briefly, PP-50 and AF647 were conjugated using the coupling reactants N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (sulfo-NHS). PP-50 was dissolved in a mixture of DMSO and water. Subsequently, EDC and sulfo-NHS were dissolved in water. This mixture was added to the PP-50 solution and the reaction was then incubated at room temperature for 1 hour. Next, AF647 cadaverine was dissolved in 1 M Na₂CO₃ and added to the activated PP-50/EDC/sulfo-NHS mixture to react at room temperature overnight in the dark. After this incubation, 5 % DMSO in D-PBS was added to the mixture to dilute the solution and dissolve any precipitate. The final solution was dialysed for a week (MWCO 3,500 Da) and the water was changed every other day. Subsequently, the solution was transferred to a 50 ml tube and 1 M HCl was added to precipitate the labelled polymer. The solution was then centrifuged at 1,500 G for 3 min to recover a blue solid. This precipitate was dissolved in 1 M NaOH and D-PBS and the solution was then dialysed against water for 3 hours and lyophilised using a Fisher Scientific Heto LyoLab3000 (UK) to yield a blue solid powder.

PP-50/AF647 was analysed by using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy to determine whether PP-50 were successfully conjugated with Alexa Fluor 647 cadaverine. Spectra were collected using a Thermo Nicolet Nexus 870 spectrometer (Waltham, MA, USA) as the average of 32 scans with a wavenumber resolution of 4 cm⁻¹ in the 600-4000 cm⁻¹ range.

2.3. Cell culture

SAOS-2 osteosarcoma cells were cultured in DMEM with FBS (10 % v/v), L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 µg/ml) in 75 cm² flasks supplied by Corning (UK). Cells were incubated in a 37 °C incubator with 5 % of CO₂. At 70 %

confluence, cells were washed twice with PBS, then subcultured with trypsin (0.05 % w/v) and EDTA (0.02 % w/v) and subsequently replated for further expansion or experiments.

2.4. Colocalisation of PP-50 with endocytosis tracers

Cells were seeded on 1.8 cm² chambered cell culture cover glasses (Nunc, UK) at a density of 3×10^4 cells/well. Cells were grown for 24-48 hours and then incubated for 2 hours with 100 µg/ml transferrin-AF488, 2 µM lacCer-BODIPY or 1 mg/ml fluorescein isothiocyanate–dextran (150 kDa) in the presence of 250 µg/ml PP-50/AF647. Subsequently, cells were incubated for 15 minutes with 5 µg/ml Hoechst 33342; this permeable dye is used to evaluate nuclear morphology as it emits fluorescence when bound to DNA. Cells were washed twice with PBS and extracellular labelled molecules were quenched using 0.4 % trypan blue [20]. Cells were washed again with PBS and left in growth media. Cells were analysed using a TCS SP5 inverted laser scanning microscope (Leica, Germany). An argon laser was used to visualise transferrin-AF488, lacCer-BODIPY, and fluorescein isothiocyanate–dextran (emission at 488 nm and an emission filter set at 505-555 nm). To visualise PP-50/AF647, a helium neon laser was used (emission at 633 nm and emission filter set at 650-700 nm). H33342 stained nuclei were excited using a diode laser emitting at 405 nm. Images were taken sequentially.

2.5. Image analysis

Fluorescent images of cells incubated with labelled transferrin, lacCer or dextran in the presence of labelled PP-50 were acquired using the appropriate wavelengths for each molecule, as explained above. Images were then merged and converted to 8 bit RGB images using ImageJ software. Analysis with the JACoP plugin provided Manders' overlap coefficient.

2.6. PP-50 mediated transport of endocytosis tracers

Cells were passaged into 24-well plates at a density of 5×10^4 cells/well and grown for 24-48 hours. Cells were then washed with PBS and incubated with 100 µg/ml transferrin-AF488, 2 µM lacCer-BODIPY or 1 mg/ml fluorescein isothiocyanate–dextran, in the presence and absence of 250 µg/ml PP-50 for 2 hours. The uptake of endocytosis tracers was assessed using a FACScan flow cytometer (Becton Dickinson, USA). Data were acquired using Cellquest software and analysed using the Beckman Coulter Summit software.

2.7. Toxicity screening of endocytosis inhibitors

Cells were passaged into 96-well plates at a density of 5×10^3 cells/well and grown for 24 hours. Cells were then washed with PBS and incubated with various concentrations of m β cd, nystatin, sucrose, and rottlerin for 2 hours. The toxicity was evaluated by quantifying metabolic activity using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) according to the manufacturer's instructions. Absorbance was measured at 490 nm using a 96-well plate reader (BMG Labtech, UK) and corrected by subtraction of background absorbance.

2.8. Pre-treatment with endocytosis inhibitors

For inhibition studies, 5×10^4 cells/well were seeded in 24-well plates and grown for 24-48 hours. Cells were subsequently pre-incubated for 1 hour with CavME inhibitors 5 mM m β cd and 250 μ g/ml nystatin, CDE endocytosis inhibitor 300 mM sucrose, and MPC inhibitor 2.5 μ M rottlerin. Calcein, a non-permeable hydrophilic dye which accounts for the activity of cytoplasmic esterases, was used to stain cells capable of incorporating it into their cytoplasm [18]. Cells were then incubated for 1 hour at 37 °C with 2 mM calcein and 250 μ g/ml PP-50. A different treatment consisted of cells incubated for 1 hour with 2 mM calcein and 250 μ g/ml PP-50 at 4 °C. For calcein uptake quantification, cells were analysed using a FACScan flow cytometer (Becton Dickinson, USA). Data were acquired using Cellquest software and analysed using the Beckman Coulter Summit software.

2.9. Colocalisation of PP-50 with endosomal pathway markers

Cells were seeded on 1.8 cm² chambered cell culture cover glasses (Nunc, UK) at a density of 3×10^4 cells/well and grown for 24-48 hours. Cells were then transduced with CellLight reagents according to the manufacturer's instructions. Briefly, cells were incubated for 16 hours with 30 μ l of CellLight solution with baculovirus containing either early endosomes Rab5a-GFP, late endosomes Rab7a-GFP or lysosomes Lamp-1-GFP. After this period, cells were washed twice with PBS, incubated with 250 μ g/ml PP-50/AF647 for 2 and 24 hours and finally incubated for 15 minutes with 5 μ g/ml H33342. Cells were washed twice with PBS and extracellular labelled molecules were quenched using 0.4 % trypan blue. Cells were washed again with PBS and left in growth media. Cells were analysed using a TCS SP5 inverted laser scanning microscope (Leica, Germany). An argon laser was used to visualise the GFP labelled early endosomes, late endosomes and lysosomes (excitation at 488 nm and emission filter set at 505-555 nm). To visualise PP-50/AF647, a helium neon laser was

used (excitation at 633 nm and emission filter set at 650-700 nm). H33342 stained nuclei were excited using a diode laser emitting at 405 nm, with an emission filter set between 425 nm and 475 nm.

2.10. Statistical analysis

All measurements were carried out by triplicate in three different replicates. Flow cytometry results were analysed using a one-way ANOVA followed by a Tukey's test for multiple comparisons. The tests were analysed using GraphPad Prism (GraphPad Software, US). The difference was considered statistically significant when $p < 0.05$.

3. Results

3.1 Colocalisation of PP-50 with endocytosis tracers

The colocalisation of PP-50 with endocytosis tracers was analysed by confocal microscopy. Cells were incubated with labelled PP-50 (red fluorescence) and either with labelled transferrin, lacCer, or dextran (green fluorescence) (Fig. 1). The colocalisation of these endocytosis tracers with PP-50 is shown as orange. As seen in the figure, osteosarcoma cells readily incorporated PP-50/AF647 intracellularly. The labelled polymer appeared in the cytoplasm, located mainly in the perinuclear space. Endocytosis tracers were also transported across the cell membrane, as confirmed by intracellular staining of cells. A higher colocalisation was observed with transferrin and lacCer, with both tracers accumulated in sites close to the cell nucleus. On the other hand, dextran intracellular signal was lower when compared to transferrin or lacCer. Cells also displayed normal nuclei features, shown by the staining with Hoechst H33342.

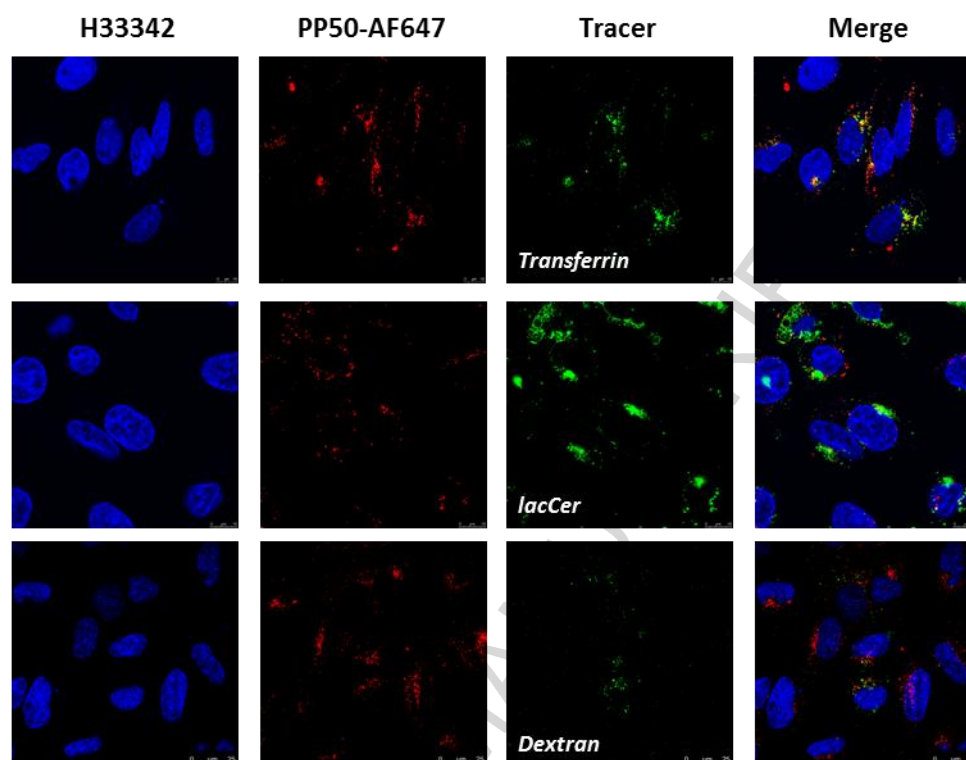


Figure 1: PP-50/AF647 colocalisation analysis with endocytosis tracers on SAOS-2 by confocal microscopy. Cells were incubated with transferrin-488, lacCer-BODIPY or dextran-fluorescein isothiocyanate (green fluorescence) and 250 $\mu\text{g/ml}$ PP-50/AF647 for 2 hrs (red fluorescence). Subsequently cells were stained with 5 $\mu\text{g/ml}$ Hoechst H33342 (blue fluorescence) for 15 min. Data are representative of a minimum of three separate experiments.

3.2 Image analysis of PP-50 colocalisation with endocytosis tracers

The analysis of the colocalisation PP-50 with endocytosis tracers was assessed by using ImageJ software and at least 5 independent images for each marker. The Manders' overlap coefficient was derived with the JaCoP ImageJ tool. The coefficient was 0.548 ± 0.048 for CDE, 0.471 ± 0.067 for CavME, and 0.336 ± 0.078 for MPC.

3.3. PP-50 mediated transport of endocytosis tracers

To assess whether PP-50 was directly involved in the transport of endocytosis tracers, cells were incubated with transferrin, lacCer, and dextran in the presence and absence of PP-50. The uptake of these tracers was investigated using flow cytometry (Fig. 2). As seen in Fig. 2, a significant uptake increase of transferrin ($10 \pm 4 \%$) and dextran ($36 \pm 15 \%$) was obtained in the presence of PP-50. No significant difference was observed for lacCer in the presence of PP-50.

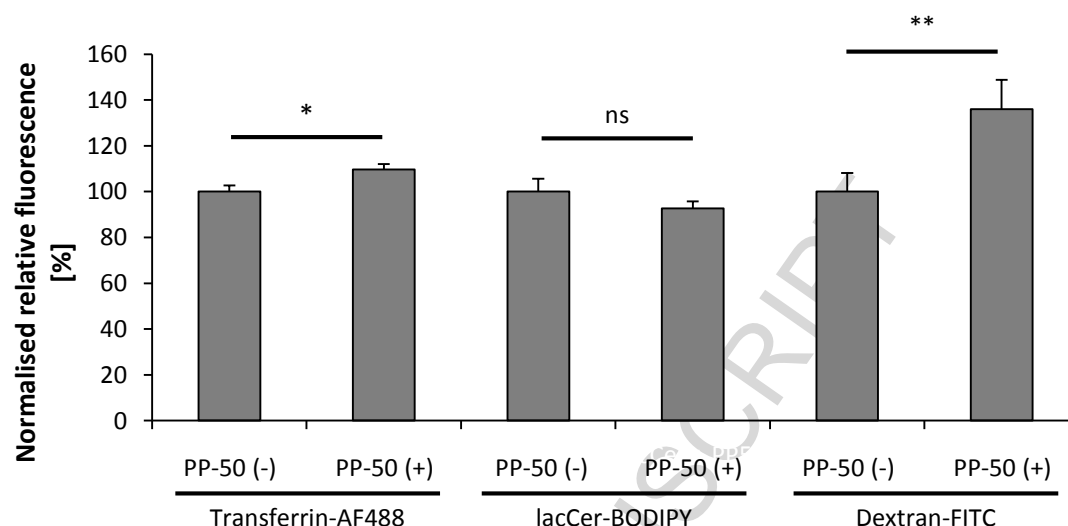


Figure 2: Flow cytometry analysis of endocytosis tracers' uptake on SAOS-2 in the presence or absence of PP-50. Cells were incubated with transferrin-488, lacCer-BODIPY or dextran-FITC (green fluorescence) in the presence or absence of 250 μ g/ml PP-50/AF647 for 2 hrs. Fluorescence was obtained using a FACScan flow cytometer. Data are representative of a minimum of 3 separate experiments. Data were normalised to PP-50 (-) from each tracer. * $P < 0.05$, ** $P < 0.01$. NS represents no significant difference.

3.4. Metabolic activity analysis

To analyse different endocytosis inhibitors involved in PP-50 mediated transport, the effect on metabolic activity of cells incubated with these molecules was previously investigated using the MTS assay (Fig. 3). The cytotoxicity was examined as a function of the inhibitors concentration within a 2 hour period. As seen in Fig. 3, nystatin and sucrose concentrations were not cytotoxic and were well tolerated by the cells. M β cd and rottlerin showed a significant decrease in metabolic activity at concentrations higher than 10 mM and 5 mM, respectively. Concentrations of 5 mM m β cd, 2.5 μ M rottlerin, 250 μ g/ml nystatin, and 300 mM sucrose were chosen to minimize their cytotoxic effects.

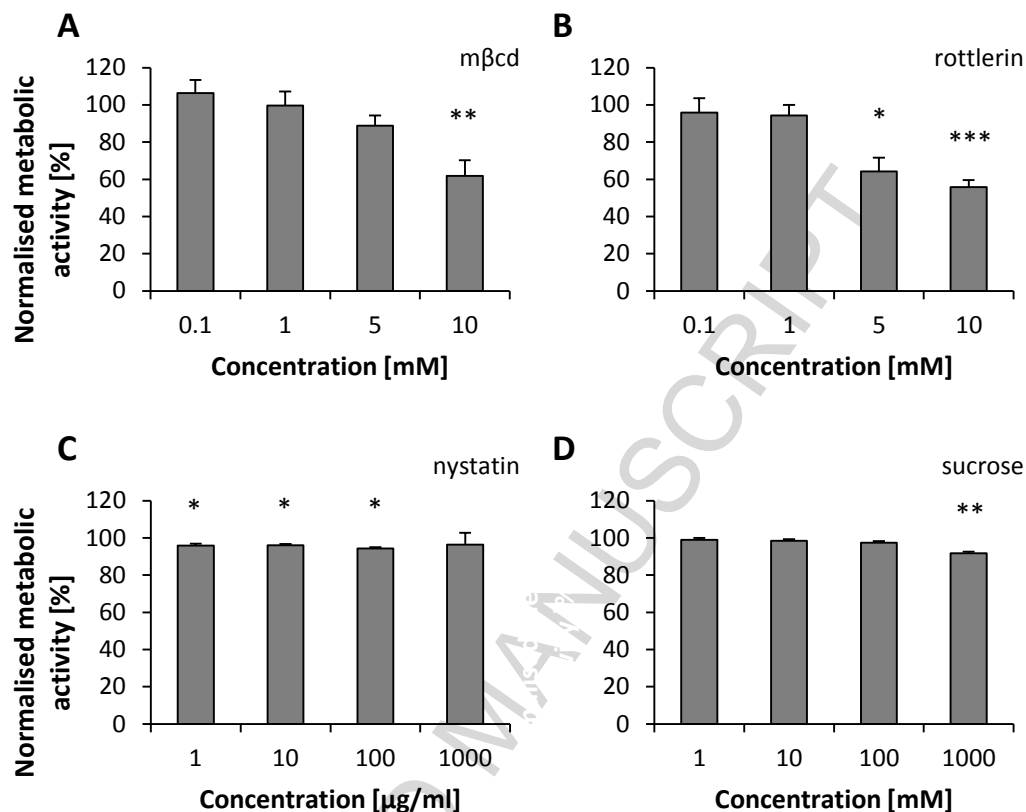


Figure 3: Metabolic activity of SAOS-2 cells after incubation for 2 hrs with different endocytosis inhibitors. A) Mβcd. B) Rottlerin. C) Nystatin. D) Sucrose. Data was normalised to cells incubated in the absence of the inhibitor. Data were derived from three replicates. Error bars represent standard error. Data were normalised to untreated cells. * P<0.05, ** P<0.01, *** P<0.001.

3.5. PP-50 mediated calcein uptake after pre-treatment with endocytosis inhibitors

To investigate the cell internalisation pathway of PP-50, endocytosis inhibition studies were conducted using flow cytometry. The inhibition of calcein uptake (a non-permeable dye) mediated by PP-50 was assessed by pre-treating cells with different endocytosis inhibitors (Fig. 4). As seen in Fig. 4, reduced levels of calcein uptake were seen when using CDE, CavME and MPC inhibitors. A significant decrease in calcein uptake compared to the control was found when cells were pre-incubated with mβcd (67 ± 5 %), nystatin (54 ± 14 %), sucrose (34 ± 4 %), and rottlerin (26 ± 10 %). Cells incubated at 4 °C also showed a significant decrease in calcein uptake (87 ± 4 %), with similar levels as those obtained with untreated cells.

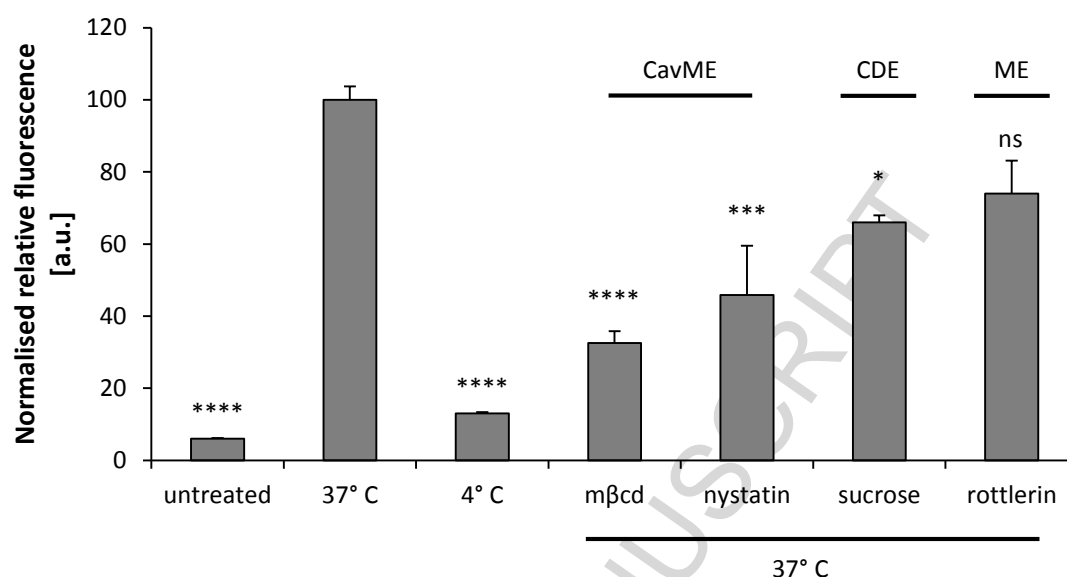


Figure 4: Flow cytometry analysis of calcein uptake of SAOS-2 cells incubated with different endocytosis inhibitors in the presence of PP-50. Cells were first incubated for 1 hour with either 5 mM mβcd, 250 μg/ml nystatin, 300 mM sucrose, or 2.5 μM rottlerin. Cells were then incubated with 2 mM calcein and PP-50 (250 μg/ml) at 37 °C for 1 hour. A different treatment consisted of cells incubated with 2 mM calcein and PP-50 (250 μg/ml) at 4 °C. Error bars represent the standard error. Data are representative of a minimum of three separate experiments. CDE, CavME and MPC represent clathrin and caveolin-mediated endocytosis and macropinocytosis, respectively. Asterisks represent statistical significance compared to 37° C. *P<0.05, ***P<0.001, ****P<0.0001. NS represents no significant difference.

3.6. Colocalisation of PP-50 with endolysosomal pathway markers

The colocalisation of PP-50 with endolysosomal pathway markers was analysed by confocal microscopy in order to identify the fate of PP-50 when internalised. Cells were transduced with CellLight endosomes, late endosomes or lysosomes to produce GFP-labelled Rab5a, Rab7a, and Lamp-1, respectively. Cells were then incubated in the presence of PP-50/AF647 for 2 and 24 hours (Fig. 5). Transient expression of endolysosomal proteins was obtained in osteosarcoma cells using CellLight reagents.

After 2 hours of incubation, a small degree of colocalisation was observed between PP-50/AF647 and the early endosome marker GFP-Rab5a. A higher degree of colocalisation was also observed with the late endosome marker Rab7a, although most of the polymer was able to escape the endolysosomal pathway before this incubation period (Fig. 5, upper panel). No PP-50/AF647 colocalisation was observed with the lysosome marker Lamp-1 after 2 hrs. For a 24 hour incubation, no significant colocalisation was observed with either of the endolysosomal markers (Fig. 5, lower panel).

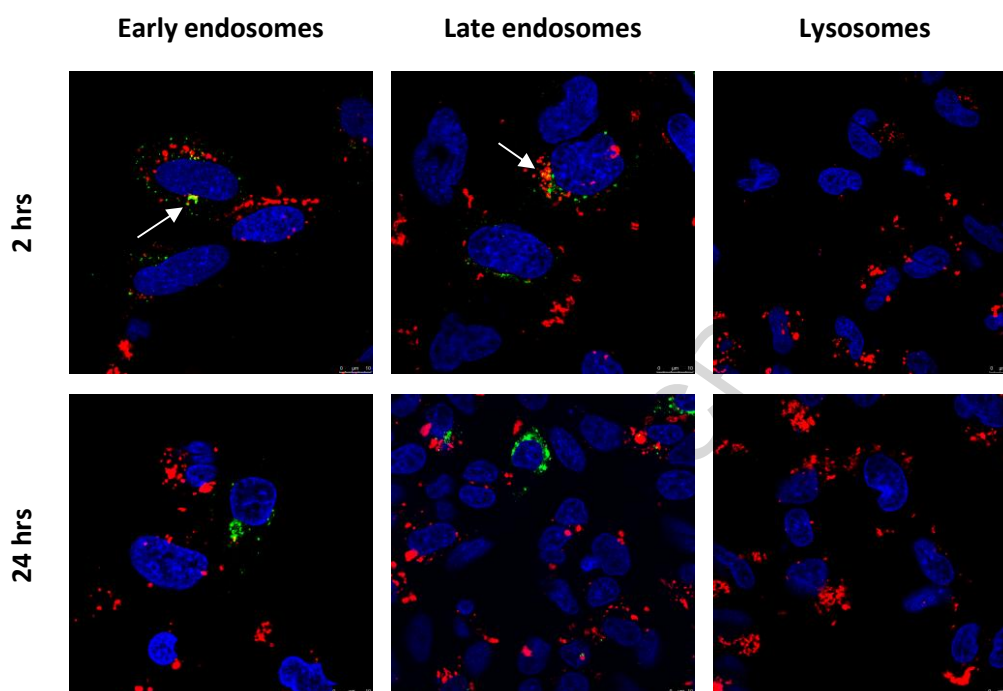


Figure 5: Confocal microscopy analysis of PP-50/AF647 colocalisation with markers for the endosomal pathway on SAOS-2. Cells were first transduced with CellLight reagents (early and late endosomes and lysosomes) and after 24 hrs cells were incubated for 2 and 24 hrs with PP50-AF647 (250 $\mu\text{g}/\text{ml}$) (red fluorescence). Subsequently cells were stained with 5 $\mu\text{g}/\text{ml}$ Hoechst H33342 (blue fluorescence) for 15 min. Data are representative of a minimum of three separate experiments. Arrows indicate colocalisation.

4. Discussion

In this study, the trafficking of PP-50 through the cell membrane was investigated in SAOS-2 cells. Analysis of colocalisation with endocytosis tracers, inhibition of endosomal routes, as well as colocalisation with endolysosomal pathways markers showed that PP-50 is able to penetrate cell membranes, with the majority of the polymer escaping the endolysosomal pathway.

To determine the internalisation pathway of PP-50, colocalisation of the polymer was investigated using molecules known to be taken up by specific endocytic routes: 1) transferrin, a CDE tracer; 2) lactosylceramide, a CIE tracer; and 3) dextran, a macropinocytosis tracer (Fig. 1) [21–23]. Usage of endocytic routes was quantitatively analysed using the Manders coefficient. The Manders coefficient compares the fluorescence of each pixel from two independent images and is almost independent of signal proportionality; it is primarily sensitive to co-occurrence, regardless of signal levels [25]. This number shows the fraction of transferrin, lacCer or dextran colocalised with the polymer, and varies from 0 for non-overlapping images to 1 for complete colocalisation [24,25]. The

coefficient was close to 0.5 for both transferrin and lacCer, and 0.336 ± 0.078 for dextran, suggesting that PP-50 is partially transported by CDE and CIE, and may also be trafficked by macropinocytosis to a lesser extent [26]. Both confocal visualisation and quantitative analysis of the colocalisation were in agreement, strongly indicating the involvement of cell receptor-based endocytosis for cellular uptake of PP-50.

To investigate whether PP-50 directly induced additional transport of endocytosis tracers, transferrin, lacCer, and dextran were incubated in the presence and absence of the polymer and their uptake was assessed by flow cytometry (Fig. 2). The largest significant difference in uptake when incubated with the polymer was obtained for dextran, as PP-50 increased its uptake by over 35 %. This increase in dextran transport, a polysaccharide known to undergo MPC, could be partially attributed to a change in the fluidity of the cell membrane. Membrane fluidity refers to the viscosity of the lipid bilayer or the level of lipid membrane packaging [27]. Changes in plasma membrane fluidity or its electrostatic dipole potential have been shown to increase dextran transport in previous studies [28]. Therefore, PP-50 may not only be partially using this route but also inducing the fluidity of the cell membrane.

To internalise molecules, CDE relies in clathrin-coated pits located in the cell membrane whereas CavME uses caveolin-formed vesicles, a type of lipid rafts composed of cholesterol, phospholipids or glycopospholipids [20,29–31]. On the other hand, macropinocytosis relies on actin to ingest large amounts of extracellular fluid [23]. The study of these pathways is normally investigated by selectively inhibiting different routes using pharmaceutical molecules [11,32,33]. In this work, CDE was inhibited using an hypertonic sucrose solution able to disperse clathrin lattices on the plasma membrane [34]. An inhibition of over 30 % was obtained, suggesting a participation of CDE in the trafficking of PP-50. Next, CavME inhibition was assessed using m β cd, a cyclic oligomer of glucopyranoside that acts as a cholesterol-depleting agent, and nystatin, a polyene antibiotic able to change properties of cholesterol-rich membrane domains [29,33,35]. Both m β cd and nystatin inhibited over 50 % of calcein uptake when compared to the control, indicating that CavME is partially involved in the transport of PP-50. Although inhibition of this route was accomplished, PP-50 may be using other caveolin or clathrin-independent mechanisms that also depend on lipid-rich areas of the membrane. These mechanisms have been found for different molecules such as cholera toxin B, but a distinction with CavME cannot be made at this point [36,37]. Macropinocytosis inhibition was investigated using rottlerin, a polycyclic aromatic compound, involved in the cytoskeletal reorganisation [38,39]. A 26 % inhibition of MPC was observed, indicating again the use of this pathway in a smaller degree compared to other routes. It is worth mentioning that the study of endocytosis pathways is still an evolving field that is not

fully understood. Although CDE is the most studied and frequently used for the cellular entry of internalised molecules, alternative mechanisms such as CIE are equally important [6,22,31].

Although CDE, CavME, and macropinocytosis were inhibited partially, none of the pharmaceutical endocytic inhibitors was able to fully inhibit PP-50 mediated calcein uptake. Other compounds were also used to inhibit endocytosis, such as genistein and chlorpromazine, but no significant difference in calcein uptake was found (data not shown) [40,41]. These results indicate that PP-50 is internalised through multiple endocytic pathways within a specific cell type, as commonly seen with most cell-penetrating peptides that rely on a combination of mechanisms rather than a single one [40,42]. In addition, this process appears to be temperature-dependent as seen by the inhibition of calcein uptake at 4 °C, suggesting an active mechanism of internalisation [35].

In order to further characterise the intracellular fate of PP-50, cells were transduced with endosomes and lysosomes markers. Time points of 2 and 24 hours were chosen to ensure the visualisation of PP-50's escape from the endolysosomal route, as previous studies have shown that endocytosis is a fast process [13]. Representative images of the colocalisation from the centre plane of the cells were obtained by confocal microscopy. After 2 hours of incubation with PP-50, confocal images suggest that most of the polymer has already escaped the lysosomal route, as evidence by the red and green staining rather than yellow. It was also observed that the polymer accumulated in the perinuclear region of cells. As PP-50 mimics viral peptides that are able to penetrate cell, this is in agreement with the fate of DNA viruses that localise in this region to deposit their genomic DNA in the host nucleus [43–45]. After 24 hours of incubation with PP-50, little, if any of the polymer colocalised with these markers. Thus, PP-50 is likely to traffic through endosomes and lysosomes prior to the incubation time used in this experiment.

The issue of biomolecular drug delivery into the cytoplasm is particularly important in order to reach eventual intracellular molecular targets and compartments. The choice of PP-50 as a drug delivery system has shown that amphipathic pH-responsive biopolymers are able to escape from lysosomes, an environment for proteolysis and degradation, by the disruption of endolysosomal membranes [7]. This is a key characteristic for the delivery of therapeutic molecules across the cell membrane.

Together, these findings suggest that PP-50 was able to escape from lysosomes, an environment for proteolysis and degradation, by the disruption of endolysosomal membranes [7]. This is a key characteristic for the delivery of therapeutic molecules across the cell membrane.

5. Conclusions

In this study, the cellular trafficking of an amphipathic pH-responsive biopolymer was studied. PP-50 colocalisation with different endocytosis tracers as well as quantitative image analysis showed that polymer cell internalisation relies mainly on clathrin and caveolin-mediated endocytosis and to a lesser extent on macropinocytosis. PP-50-mediated uptake of calcein decreased when using CDE, CIE, and MPC inhibitors, also suggesting that the polymer relies on a combination of mechanisms rather than a single one for cell internalisation. Endosome and lysosome marker colocalisation with PP-50 showed that the polymer is able to escape the endolysosomal route in less than 2 hours. Further work is needed to determine the trafficking of PP-50 in different cell systems, such as suspension cells or *in vivo* models. This study provides an important step toward the use of amphipathic biopolymers for cryopreservation and biomedical applications.

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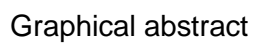
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Highlights

- Endocytosis mechanisms of an amphipathic biopolymer, PP-50, were evaluated in osteosarcoma cells
- Colocalisation of PP-50 with different endolysosomal route markers was analysed
- PP-50 relies on a combination of mechanisms rather than a single one for cell internalisation
- PP-50 was shown to escape the endolysosomal compartment before maturation